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Structure guided design of improved anti-proliferative rapalogs through biosynthetic medicinal chemistry

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General chemical methods

All solvents used were HPLC grade. LCMS/MS analysis was performed on an Agilent HP1100 HPLC system in combination with a Bruker Daltonics Esquire 3000+ ion trap mass spectrometer fitted with an electrospray source. The MS was operated in both positive and negative ion modes. UV analysis was performed at 210, 258 and 280 nm on an Agilent DAD detector. High resolution mass spectra were acquired on a Bruker BioApex II FTICR mass spectrometer or a Thermo-Fisher Finnigan LTQ Mass Spectrometer. The samples were run by Positive Ion Electrospray. NMR spectra were recorded on a Bruker Avance 500 spectrometer at 298 K operating at 500 MHz and 125 MHz for ^{1}H and ^{13}C respectively. Standard Bruker pulse programs were used to acquire the COSY, TOCSY, APT, HMQC and HMBC spectra. Coupling constants are given in Hertz. NMR experiments were referenced to the residual proton resonance of the solvent. Cyclohexanecarboxylic acid (CHCA) was purchased from Sigma-Aldrich. Temsirolimus was prepared from rapamycin using published procedures.[1]

Analytical chemistry methods

Fermentation samples were prepared for chemical analysis by mixing with an equal volume of methanol, shaken vigorously for 30 min and then clarified by centrifugation. The resulting supernatant was analysed by LCMS.

Chromatography for LCMS analysis was achieved over reversed-phase silica (Hypersil C_{18}-BDS, 150 x 4.6 mm column, 3 micron particle size) eluting at 1 mL/min using the following gradient: T=0 min, 55% B; T=15, 100% B. Mobile phase A: 10% acetonitrile:90% water, containing 10 mM ammonium acetate and 0.1% v/v formic acid; Mobile phase B: 90%

acetonitrile:10% water, containing 10 mM ammonium acetate and 0.1% v/v formic acid. The content of rapalogs was calculated by comparison to a standard calibration curve.

Compound purity was determined to be >95% by LCMS analysis observing the UV absorbance at three wavelengths (210, 254 and 280 nm), the MS response across the range *m/z* 100-1500 in both positive and negative modes, and by inspection of the ^{1}H and ^{13}C NMR spectra.

General biology methods

Standard molecular biology practice was generally observed.[2] Molecular biology enzymes and reagents were obtained from commercial sources. *Escherichia coli* DH10B (GibcoBRL) was grown in 2xTY medium and *E. coli* ET12567/pUZ8002 as described previously.[3] Vector pKC1139 was reported elsewhere.[4] *E. coli* transformants were typically selected for with ampicillin (100 mg/L), apramycin (50 mg/L), kanamycin (25 mg/L) or chloroamphenicol (12.5 mg/L) as appropriate. PCR products were verified by DNA sequencing, and new plasmids by restriction digests.

Bioinformatic analysis of rapamycin PKS modules

Alignments were carried out in the 14 rapamycin PKS modules (Genbank: CAA60460.1, CAA60459.1, CAA60462.1) using Edialign with default settings (http://emboss.bioinformatics.nl/), and the similarity graph (Figure 4) was generated using Plotcon (http://emboss.bioinformatics.nl/cgi-bin/emboss/plotcon) with a window size of 50.

Fermentation methods

Streptomyces rapamycinicus NRRL5491 and its derivative strains were maintained as frozen spore stocks. The media and protocols for the growth of *S. rapamycinicus* strains in Falcon

tubes (analytical fermentation) and in bioreactors (for compound isolation) have been described elsewhere.[5]

Isolation of strain BIOT-3218 (*S. rapamycinicus* MG7-9)

Generation of plasmid pALK83 (see Figure S1 below)

Primers MG308 5'-GTCCTAGGTGATGTCCCGGCAACACG-3' and MG309 5'-CACCTGCAGGCCCAACTCGGCCAGCTCGCT-3' were used to amplify a region of homology upstream of the rapamycin module 10 acyl transferase (rapAT10) (nt11693 to nt13289) using the cosmid cos26[6] as template. The 1596 bp PCR product was phosphorylated using T4 polynucleotide kinase and ligated into *Sma*I digested pUC18 which had been dephosphorylated. After transformation into *E. coli* DH10B, the plasmid pMG255-4 was isolated. The primers MG310 5'-CCTGGCCAGGAAAGACGAACACGATCCT-3' and MG311 5'-CGAAGCTTGAGCCGCTGGCGATCGTGGGA-3' were used to amplify a region of homology downstream of rapAT10 (nt14191 to nt15742) using cos26 as template. The 1551 bp PCR product was phosphorylated using T4 polynucleotide kinase and ligated into *Sma*I digested pUC18 which had been dephosphorylated. After transformation into *E. coli* DH10B, the plasmid pMG256-1 was isolated. The integrity of each insert was confirmed by DNA sequencing.

Plasmids pCJR26[7] and pMG256-1 were each transformed into *E. coli* ET12567 and unmethylated (dam^-, dcm^-, $hsdn^-$) plasmid DNA isolated. Plasmid pMG255-4 was digested with *Avr*II/*Hin*dIII and the 4.3 kbp fragment was isolated. pCJR26 was digested with *Msc*I/*Avr*II and the 1 kbp fragment comprising rapAT2 was isolated. pMG256-1 was digested with *Hin*dIII, partially digested with *Msc*I, and the approximately 1.5 kbp fragment isolated. These three DNA fragments were ligated and used to transform *E. coli* DH10B, and the

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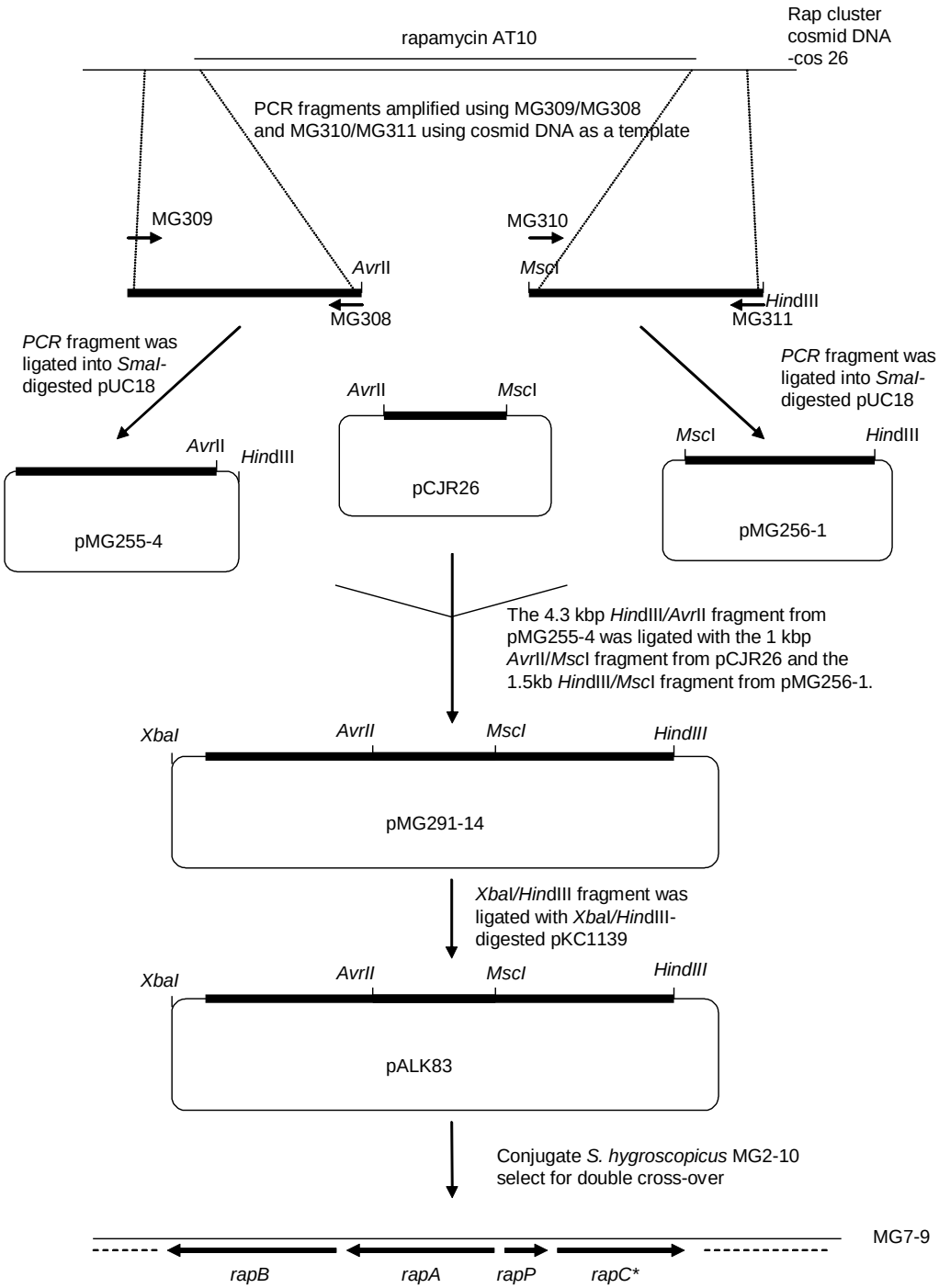
resulting plasmid pMG291-14 was isolated. This plasmid was digested with *Hin*dIII/*Xba*I and the approximately 4 kbp fragment isolated and ligated into pKC1139 digested with *Hin*dIII/*Xba*I, and the reaction mixture was used to transform *E. coli* DH10B and the resulting plasmid pALK83 was isolated.

Figure S1.



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Generation of BIOT-3218

E. coli ET12567 harbouring the plasmid pUZ8002 was transformed with pALK83 to generate the donor strain for conjugation, and was used to transform BIOT-1702. An apramycin resistant colony was isolated and patched to MMAM agar[5] containing apramycin and nalidixic acid (25 mg/L). This patch was then used to inoculate a flask containing tryptic soy broth with apramycin (50 mg/L). This culture was incubated for two days at 28 ˚C followed by two days at 37 ˚C. This culture was streaked to plates of MAMM agar containing apramycin (50 mg/L) and incubated for 7 days at 37 ˚C. Single colonies were re-patched onto the same agar and grown for a further 7 days at 37 ˚C. These patches were used to inoculate flasks of tryptic soy broth with no antibiotic, which were grown for three days at 37 ˚C. This culture was streaked to plates of MAMM agar and single colonies used to patch plates of MAMM agar with and without apramycin (50 mg/L) to look for sensitivity, representing a second recombination event involving loss of the plasmid backbone. Fourteen apramycin sensitive patches were used to inoculate Falcon tubes containing medium rapV7[5] (7 mL in 50 mL tubes) and grown for three days at 28 ˚C. These were used to inoculate Falcon tubes (0.5 mL into 7 mL of MD6[5] medium) and cultured at 26 ˚C & 300 rpm with addition of CHCA (2 mM) after 24 h. After five further days the cultures were extracted and assayed by LCMS/MS as described above. Nine separate cultures produced the same novel compound with the appropriate molecular mass for 17-desmethyl-39-deshydroxyprerapamycin (**3**). One of these cultures was selected on the basis of production level and robustness, and named BIOT-3218.

Isolation of strain BIOT-3495

Generation of plasmid pLL158

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Plasmid pSGsetrapKIJN/OMQL[8,9] was digested using *Spe*I/*Hind*III and the 8.516 kbp fragment (containing *rapKIJNOMQL*) was isolated and ligated with the integrative vector pLL150[5] similarly digested with *Spe*I/*Hind*III. After transformation into *E. coli* DH10B the final plasmid pLL158 was isolated.

Generation of BIOT-3495

E. coli ET12567 harbouring the plasmid pUZ8002 was transformed with pLL158 to generate the donor strain for conjugation, and used to transform BIOT-3218. Apramycin resistant colonies were selected on MAMM agar plates containing apramycin (50 mg/L) and nalidixic acid (25 mg/L), and then re-patched onto the same media. These patches were grown at 28°C before secondary patching onto ISP3 agar plates containing apramycin (50 mg/L) and grown for 14-21 days at 28°C. The resulting strains were assayed by analytical fermentation and extracted. The extracts were assessed for production of **4** by LCMS/MS.

Isolation of strain BIOT-3630

Generation of plasmid pLL184

Plasmid pMG260[8,9] was digested with *Spe*I/*Hind*III and the 6.3 kbp fragment (containing *rapIJNOQL*) was isolated and ligated with the integrative vector pLL150 similarly digested with *Spe*I/*Hind*III. After transformation into *E. coli* DH10B the final plasmid pLL184 was isolated.

Generation of BIOT-3630

E. coli ET12567 harbouring the plasmid pUZ8002 was transformed with pLL184 to generate the donor strain for conjugation, and used to transform BIOT-3218. Apramycin resistant

colonies were selected on MAMM agar plates containing apramycin (50 mg/L) and nalidixic acid (25 mg/L), and then re-patched onto the same media. These patches were grown at 28°C before secondary patching onto ISP3 agar plates containing apramycin (50 mg/L) and grown for 14-21 days at 28°C. The resulting strains were assayed by analytical fermentation and extracted. The extracts were assessed for production of **8** by LCMS/MS.

Isolation of strain BIOT-4125

Plasmid pALK83 was introduced into strain BIOT-4010[5] by conjugal transfer from *E. coli* ET12567 harbouring the plasmid pUZ8002 using the improved methods described recently.[5] Primary apramycin resistant recombinants were isolated and stabilised at 37 °C and then passaged three times (each 3-4 days at 37 °C) on MAMM plates without antibiotic to allow plasmid loss, and transferred to MAMM plates at 28 °C for 7-14 days to allow sporulation. Spores were harvested, serially diluted and spread onto MAMM plates to allow isolation of single colonies. These were patched to plates with and without apramycin (50 mg/L) and incubated at 28 °C; the majority of the resulting colonies had lost the antibiotic marker (and hence had undergone a secondary recombination by this procedure). To identify strains containing the target hybrid PKS, approximately ten of the strains derived from each primary recombinant were examined by analytical fermentation using standard conditions in the presence of CHCA. LCMS/MS analysis showed that secondary recombinants from only two of the eight primaries had the target phenotype, i.e. production of **10** but no production of **9**. One of these cultures was selected on the basis of production level and robustness, and named BIOT-4125.

Isolation of strain BIOT-4159

Plasmid pLL208[5] was introduced into strain BIOT-4125 by conjugal transfer, and apramycin resistant colonies selected. The resulting strains were grown under analytical fermentation conditions in the presence of CHCA and the resulting culture extracts assayed by LCMS/MS to identify the desired phenotype (enhanced production of **10**). The best strain was named BIOT-4159.

Extraction and purification of rapalogs

Typical methods have been published elsewhere.[5] The isolation of **4** is used here as representative.

The fermentation broth was stirred with an equal volume of methanol for 2 h, and then clarified by centrifugation (10 min, 3500 rpm). The supernatant was stirred with Diaion® HP20 resin (43 g/L) for 1.5 h and then filtered. The resin was washed batchwise with acetone (total volume 7.5 L) to elute **4** and the solvent removed *in vacuo*. The resulting aqueous concentrate (~800 mL) was diluted to 1 L with water and extracted with EtOAc (3×1 L). The solvent was removed *in vacuo* to give a sticky brown extract. This was dissolved in acetone (ca. 20 mL), coated onto silica, applied to a silica column (3×6.5 cm diameter) and eluted with a stepwise gradient of acetone/hexane (20 to 40%). Fractions containing **4** were pooled and the solvent removed *in vacuo*. The residue was further chromatographed over Sephadex LH20 eluting with 10:10:1 chloroform/heptane/ethanol. Fractions containing **4** were pooled and the solvent removed *in vacuo*. The residue was dissolved in acetonitrile (2.7 mL), centrifuged (10 min, 13200 rpm) and purified by reversed-phase chromatography using a Gilson HPLC, eluting a Phenomenex Luna C_{18} BDS column (21.2×250 mm, 5 micron particle size) eluting at 21 mL/min with acetonitrile:water (6:4). Pure fractions (identified by analytical HPLC) were combined and the solvent removed *in vacuo* to give **4** (133 mg).

17-desmethyl-39-deshydroxyprerapamycin (3)

A range of NMR experiments were performed in $CDCl_3$ *viz* ^{1}H, ^{13}C, APT, COSY, HMQC and HMBC. A thorough and exhaustive review of these data enabled the assignment of the majority signals for 17-desmethyl-39-deshydroxypre-rapamycin. Key was the absence of an olefinic methyl resonance immediately apparent in the ^{1}H NMR spectrum. Further, correlations between H16 and an olefinic methine not present in the spectrum of **2**[10] or its 39-deshydroxy analog,[11] could be seen in the COSY spectrum confirming the absence of a methyl group at C17. The data for the major rotamer are given below:

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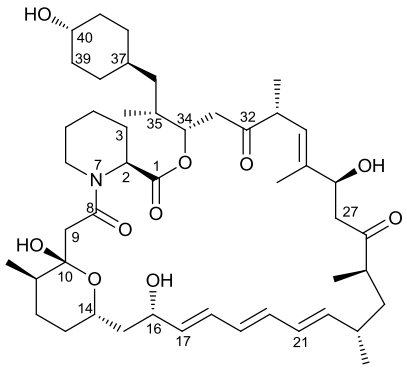
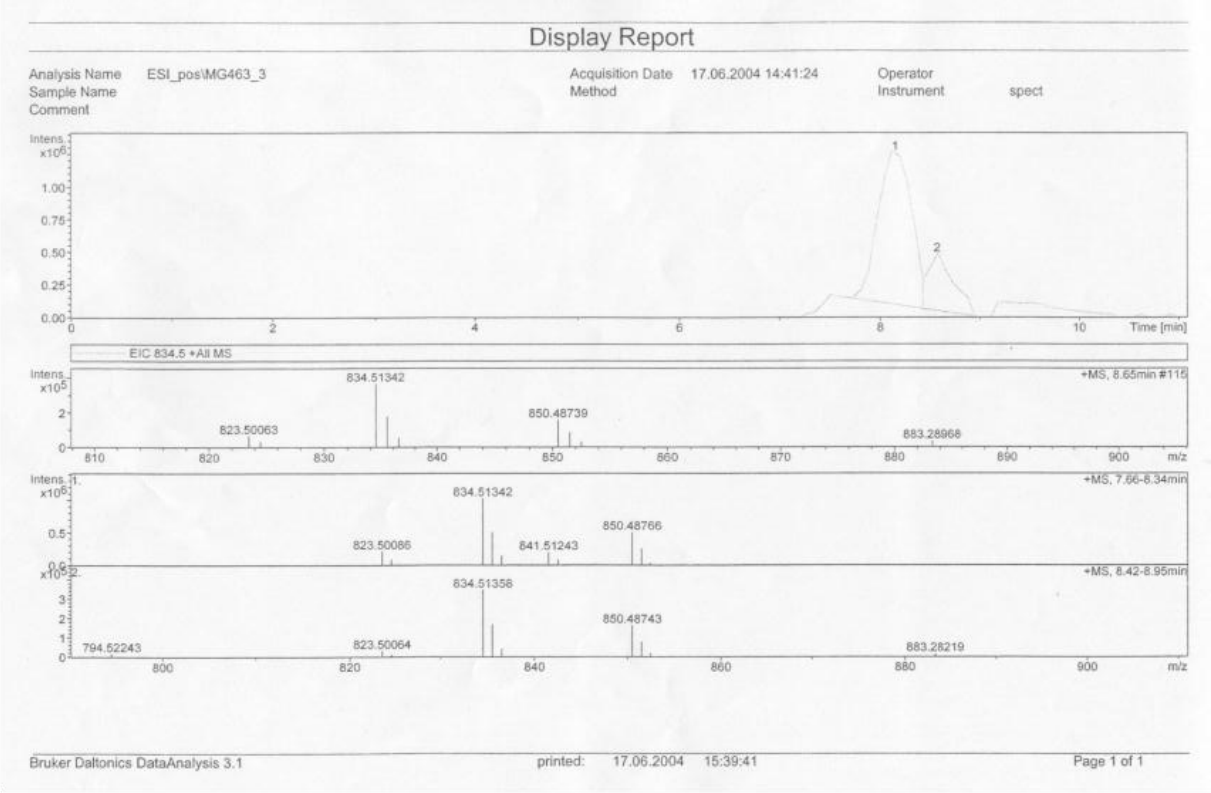


λ_{max} = 270 nm (triplet centred at 270 nm, peaks at 260 nm, 280 nm)

$C_{47}H_{73}NO_{10}Na$ calculated: 834.51319; found: 834.51342; Δ = 0.28 ppm



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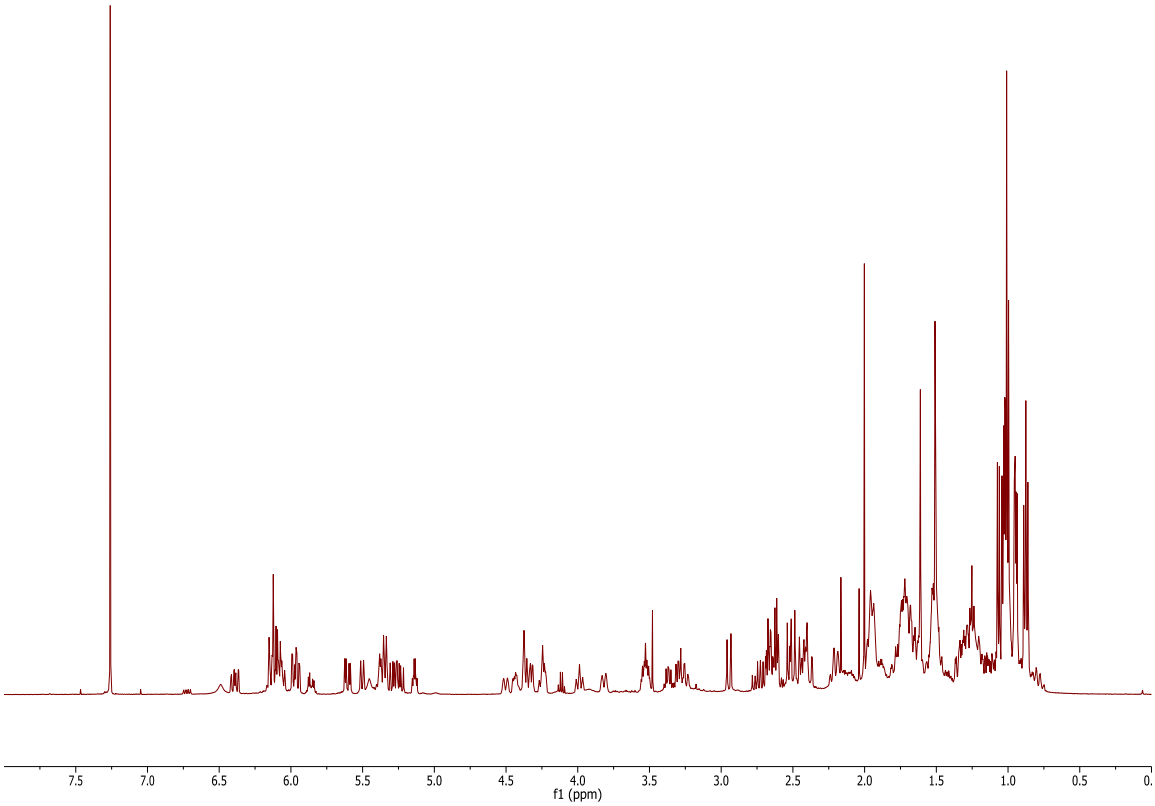
^{1}H-NMR



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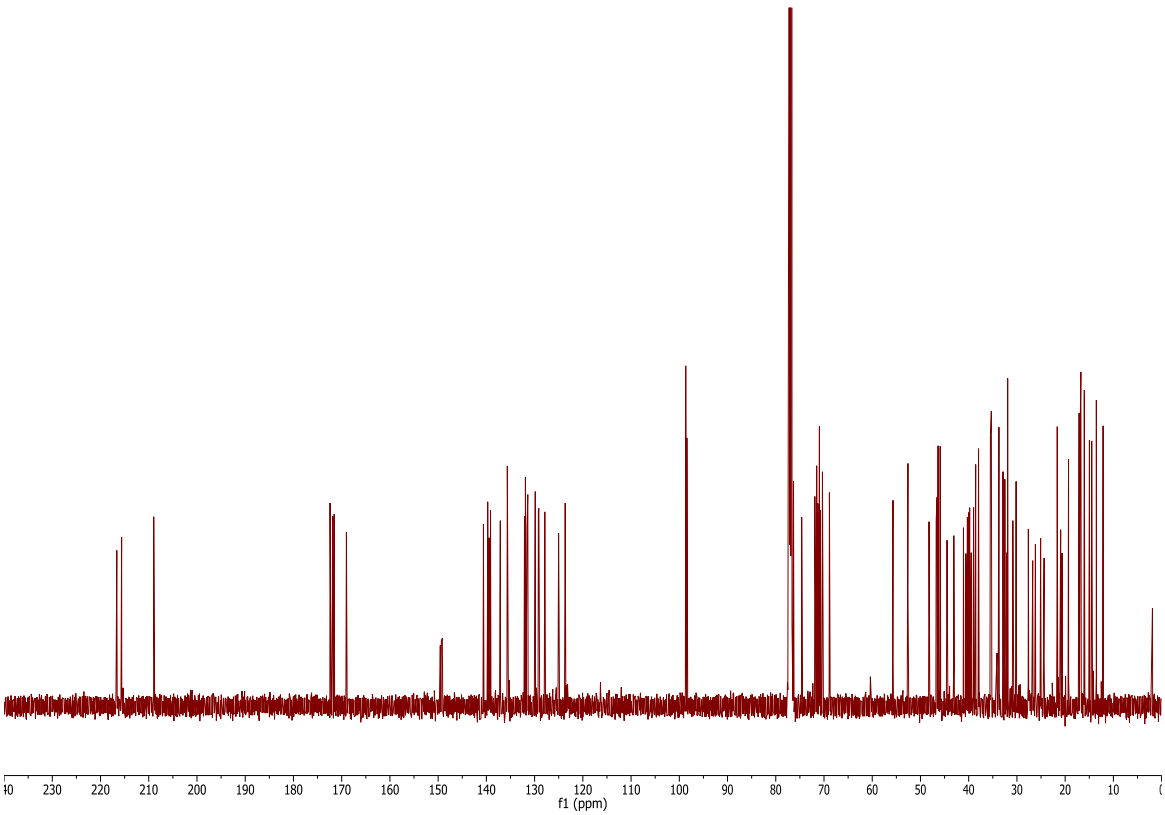

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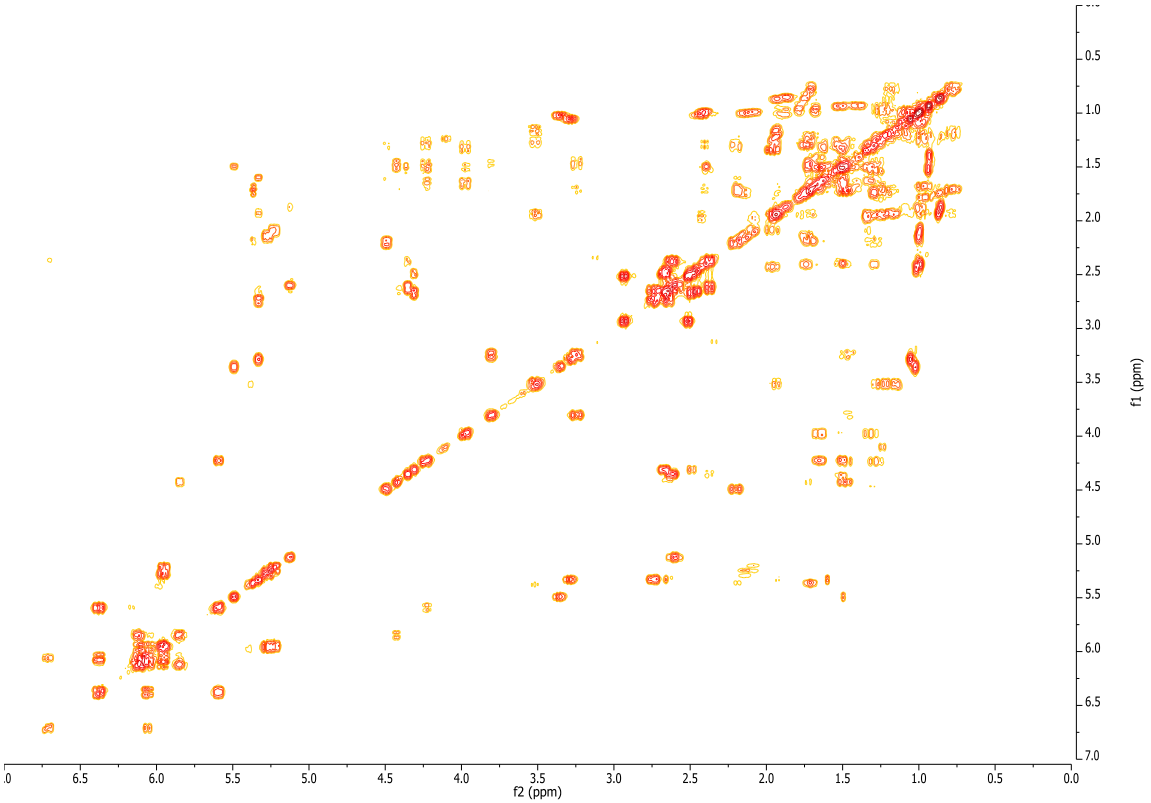
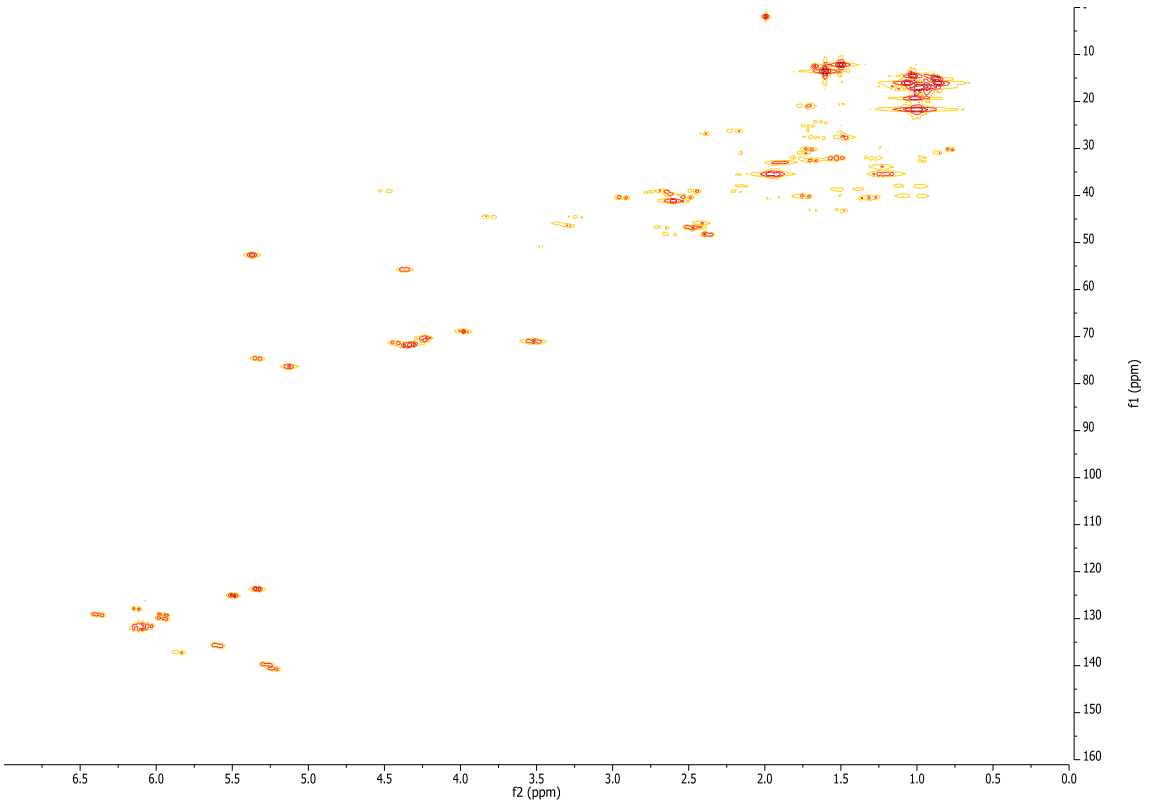
^{1}H-^{1}H-COSY



HMBC



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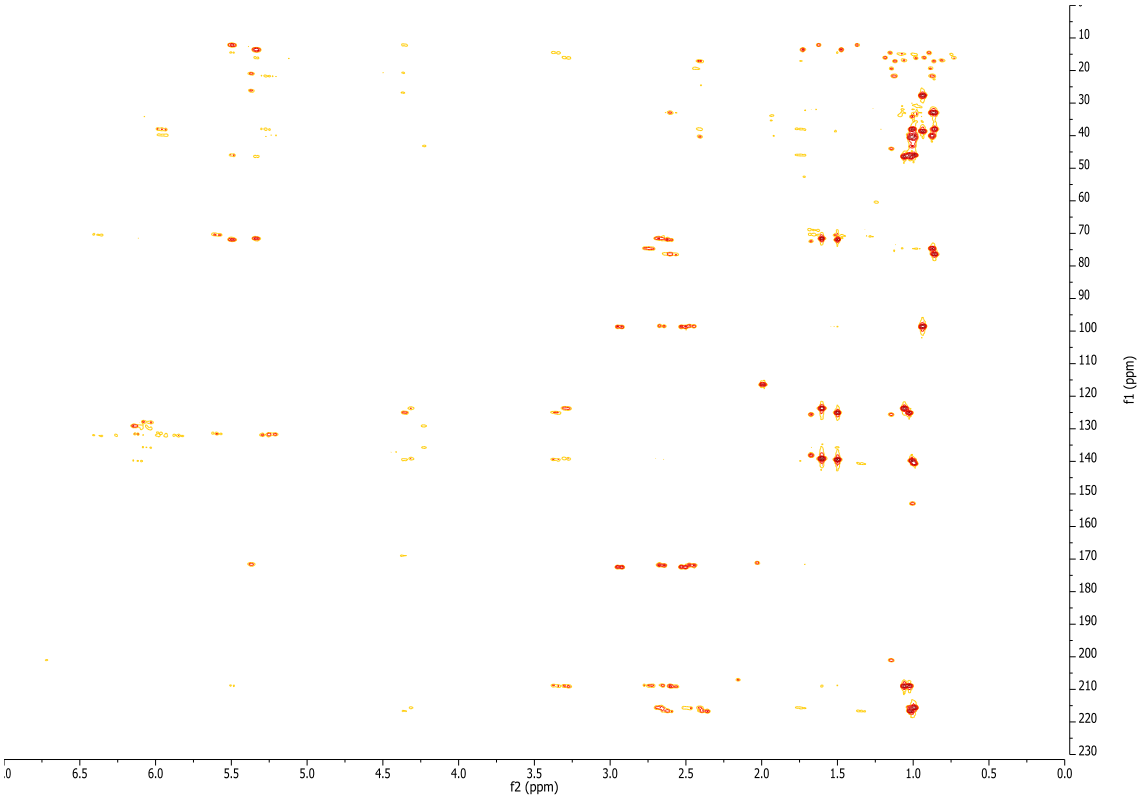
HMQC



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Position	δ_H ppm	Multiplicity, Hz	δ_C ppm
1			171.6
2	5.38	br. d, 5	52.6
3	1.73	m, complex	26.2
	2.20	m, complex	
4	1.28	m, complex	20.9
	1.73	m, complex	
5	1.48	m, complex	25.1
	1.72	m, complex	
6	3.26	m, complex	44.5
	3.82	br. d, 13.4	
7			
8			172.4
9	2.53	d, 13.4	40.4
	2.95	d, 13.7	
10			98.7
11	1.53	m, complex	38.6
11-CH_3	0.95	d, 6.4	16.7
12	1.63	m, complex	27.6
13	1.33	m, complex	31.9
	1.51	m, complex	
14	3.99	br, *pseudo* t, 12	68.9
15	1.51	m, complex	43.1
	1.66	m, complex	
16	4.24	m, complex	70.3
17	5.60	dd, 15.1, 4.8	135.7
18	6.39	ddd, 15.1, 10.1, 1.5	129.2
19	6.07	m, complex	131.4
20	6.11	m, complex	131.9
21	5.98	dd, 15.0, 9.9	129.9
22	5.28	dd, 15.0, 9.6	139.7
23	2.16	m, complex	38.0
23-CH_3	1.02	d, 6.4	21.7[d]
24	1.31	m, complex	40.0
	1.75	m, complex	
25	2.41	m, complex	45.9
25-CH_3	1.00	d, 6.7	17.1
26			215.7
27	2.50	dd, 14.2, 2	46.7
	2.68	m, complex	
28	4.32	dd, 8.5, 2.2	71.5
28-OH			
29			139.1
29-CH_3	1.61	d, 1.0	13.5
30	5.34	br. d, 10.5	123.7
31	3.30	dq, 9.7, 6.7	46.4
31-CH_3	1.07	d, 6.8	16.0
32			208.8
33	2.62	m, complex	41.1
	2.74	dd, 19.2, 10	
34	5.14	ddd, 10, 6, 4	76.3
35	1.88	m, complex	32.9
35-CH_3	0.87	d, 7.2	16.1
36	0.99	m, complex	37.9
	1.11	m, complex	
37	1.23	m, complex	33.7
38	0.79	br	30.2
	1.72	m, complex	
39	1.17	m, complex	35.2
	1.94	m, complex	
40	3.53	br. ddd	71.0
41	1.28	m, complex	35.5
42	2.03–1.11	m	32.5

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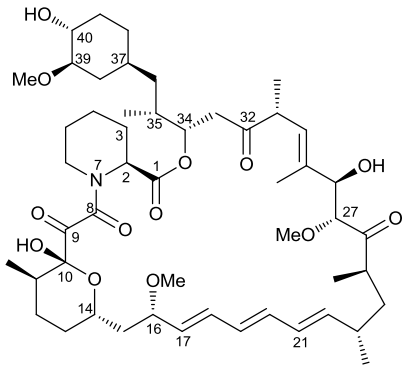
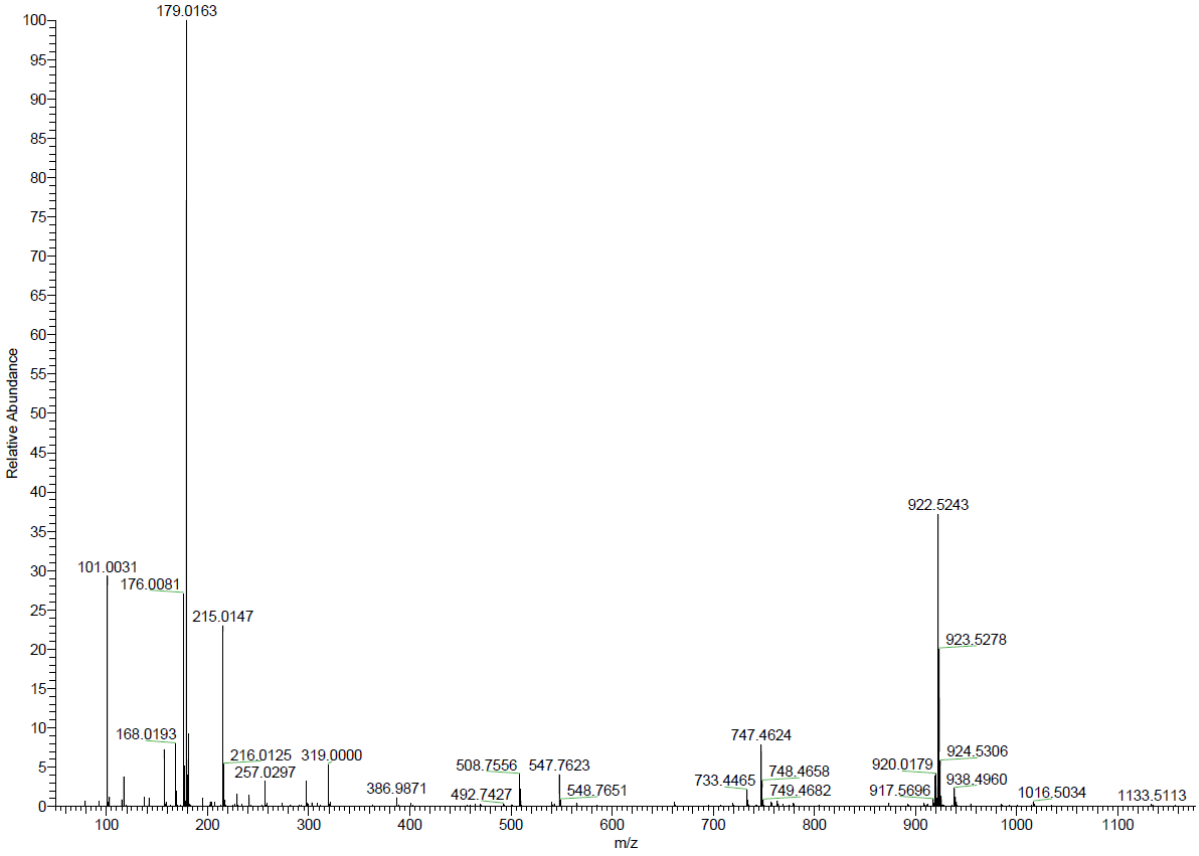
17-desmethylrapamycin (4)



λ_{max} = 270 nm (triplet centred at 270 nm, peaks at 260 nm, 280 nm)

$C_{50}H_{77}NNaO_{13}$ calculated: 922.5287; found: 922.5243; Δ = -4.74 ppm



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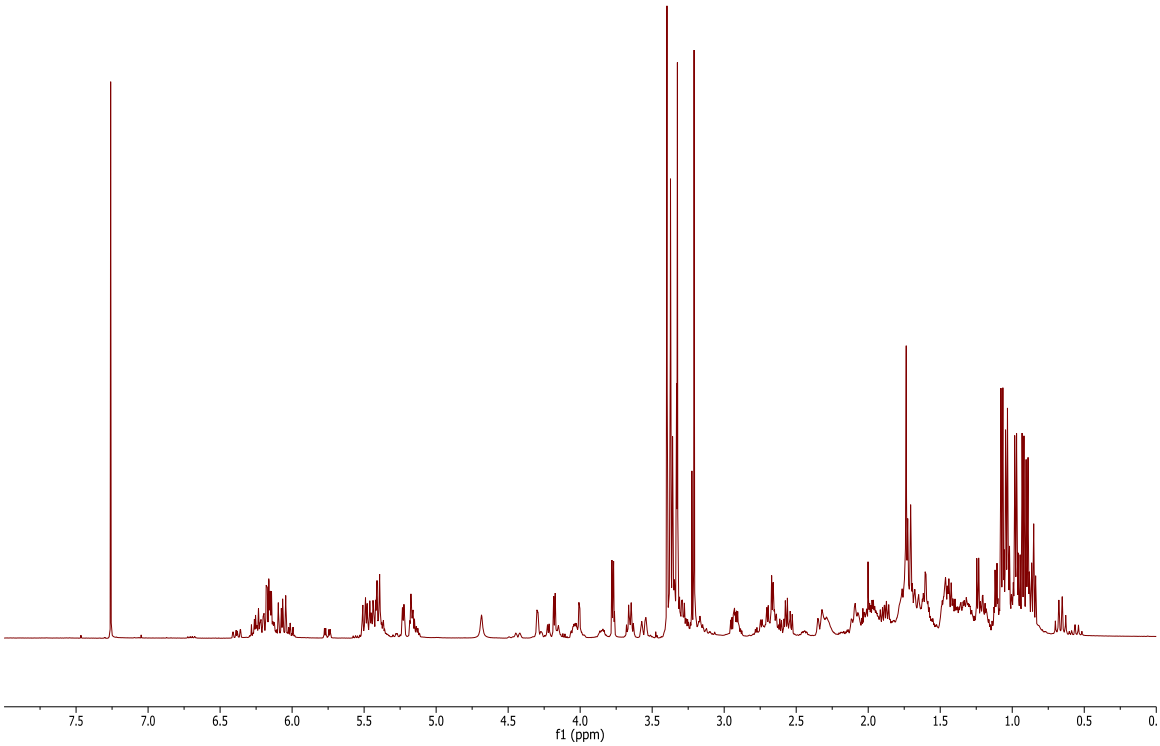
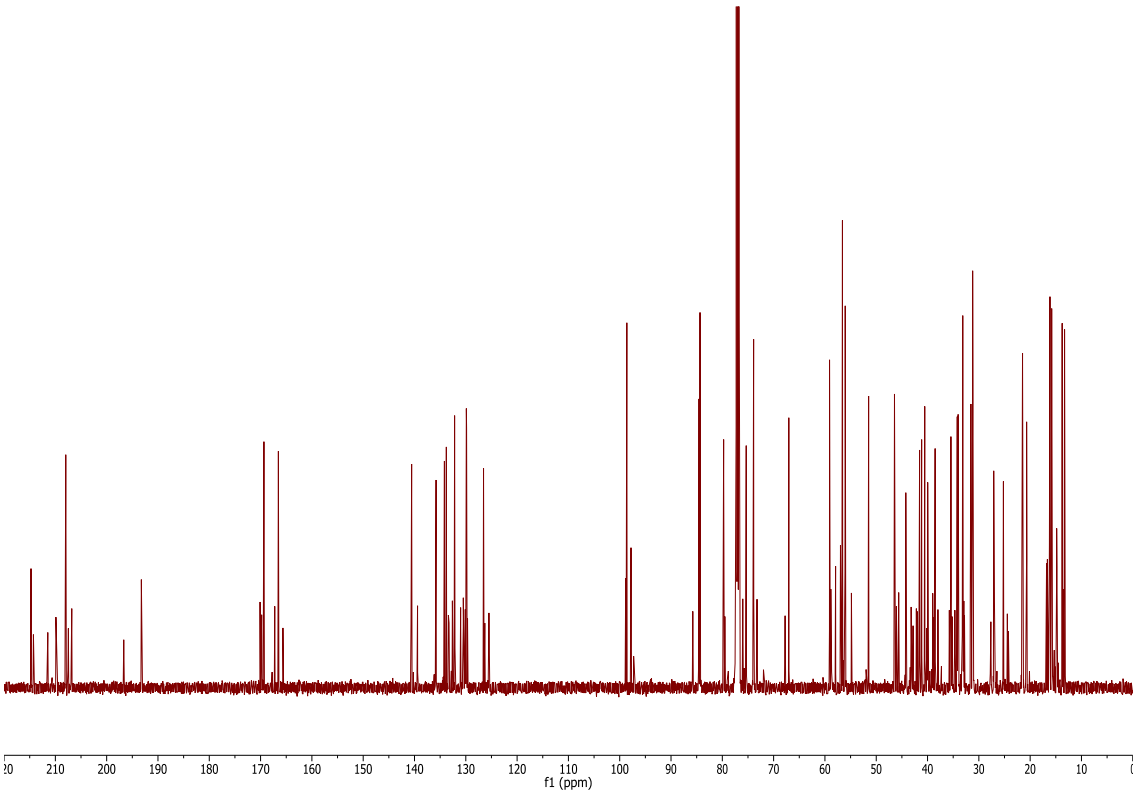
^{1}H-NMR



^{13}C-NMR



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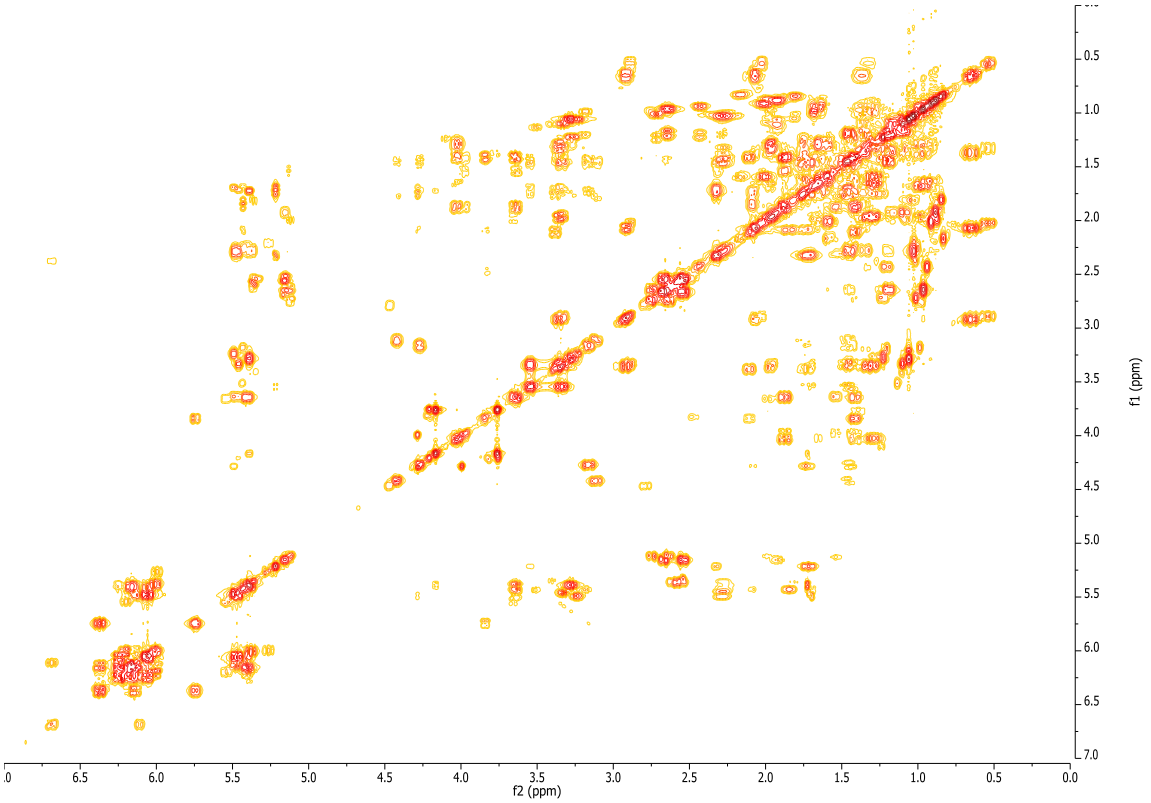
^{1}H-^{1}H-COSY



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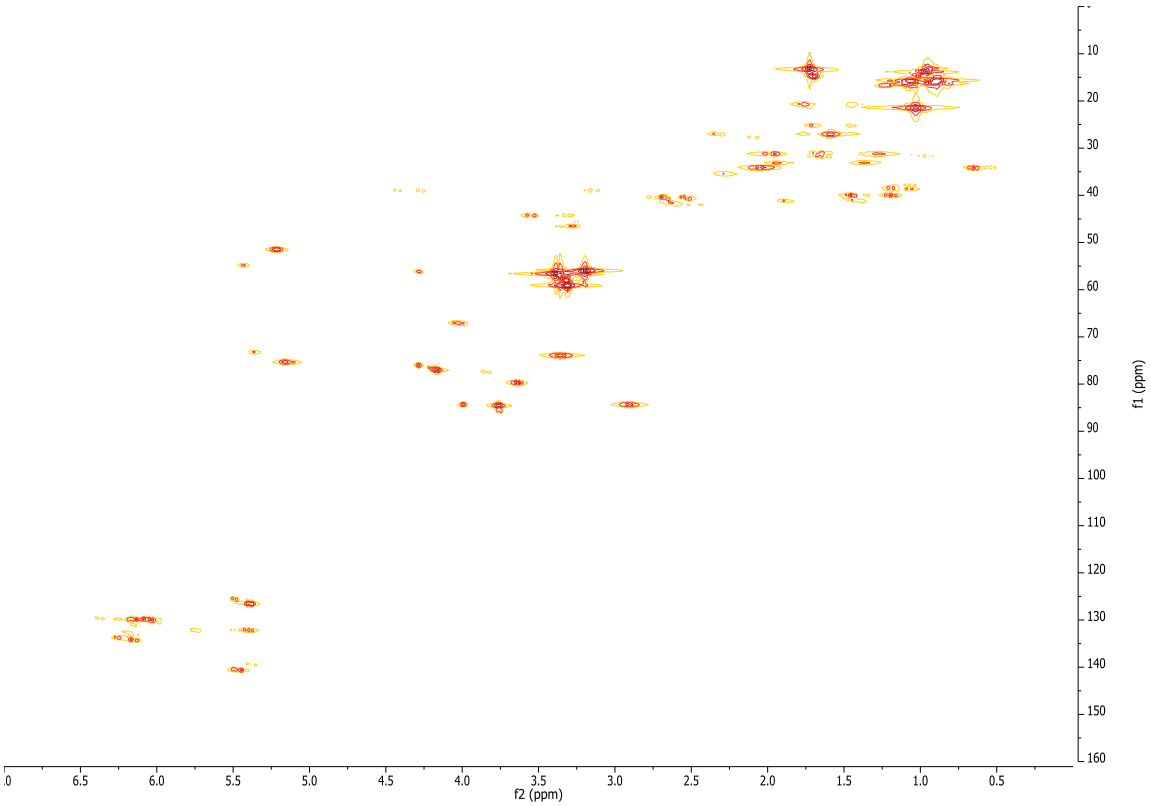

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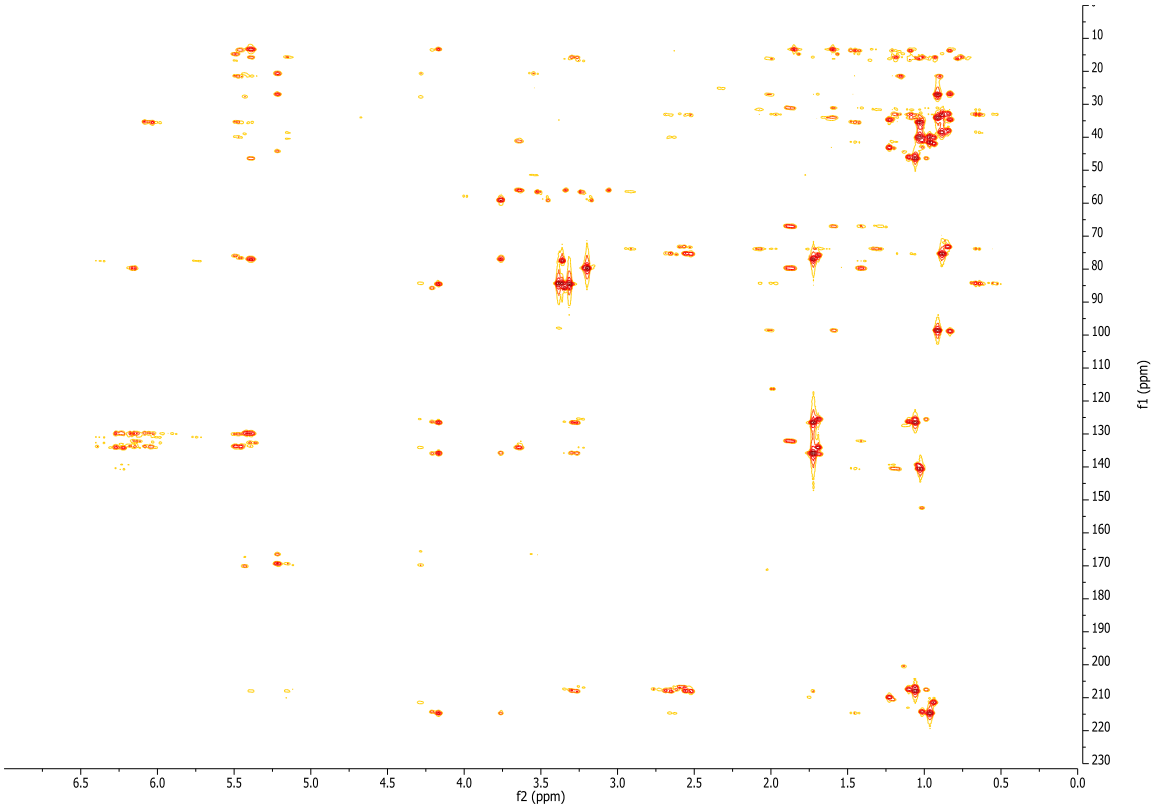

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Position	δ_H ppm	Multiplicity, Hz	δ_C ppm
1	-	-	169.3
2	5.23	br. d, 5	51.5
3	2.35	m, complex	27.1
4	1.80	m, complex	20.6
	1.46	m, complex	
5	1.75	m, complex	25.2
	1.45	m, complex	
6	2.67	ddd, 16, 10.5, 5	44.2
	2.58	ddd, 16, 9.5, 6	
7	-	-	N
8	-	-	166.5
9	-	-	193.2
10	-	-	98.6
10-OH	4.68	br. s	O
11	1.69	ddq, 11.5, 4, 6.5	33.2
11-CH_3	1.02	d, 6.5	16.2
12	1.42	m, complex	26.9
13	2.01	m, complex	31.1
	1.65	m, complex	
14	4.05	m, complex	67.0
15	1.15	ddd, 16, 10.5, 11	39.9
	1.44	ddd, 16,5.5, 6	
16	4.00	ddd, 8, 5.5, 5.5	84.4
16-OCH_3	3.34	s	55.4
17	5.50	dd, 10.5, 8	134.7
18	6.21	dd, 11, 10.5	132.2
19	6.25	dd, 14.5, 11	133.2
20	6.18	dd, 14.5, 10.5	126.5
21	6.06	dd, 15, 10.5	129.8
22	5.45	dd, 15, 8	140.5
23	2.30	m, complex	35.4
23-CH_3	0.96	d, 6.5	21.5
24	1.45	m, complex	40.5
	1.15	m, complex	
25	1.89	ddq, 10.5, 6.5, 4	41.1
25-CH_3	0.91	d, 6.5	13.7
26	-	-	214.5
27	3.77	d, 4	84.9
27-OCH_3	3.28	s	58.3
28	4.18	d, 4	75.3
28-OH	3.54	br. s	O
29	-	-	135.8
29-CH_3	1.73	s	13.2
30	5.41	d, 11	129.9
31	3.25	dq, 11, 6.5	46.4
31-CH_3	1.07	d, 6.5	15.8
32	-	-	207.9
33	2.74	dd, 17.5, 5.5	41.5
	2.52	dd, 17.5, 4	
34	5.21	ddd, 7, 5.5, 4	73.9
35	1.60	m, complex	31.1
35-CH_3	0.89	d, 6.5	15.7
36	1.60	m, complex	38.5
37	1.67	m, complex	34.0
38	2.11	ddd, 16.5, 5, 4	34.2
	1.98	ddd, 16.5, 10, 13.5	
39	2.94	ddd, 13.5, 12.5, 5	84.3
39-OCH_3	3.20	s	55.2
40	3.30	ddd, 14.4, 12.5, 6	73.9
40-OH	3.57	br. s	O
41	1.60	m, complex	31.2
42	1.78	m, complex	31.5
	1.58	m, complex	

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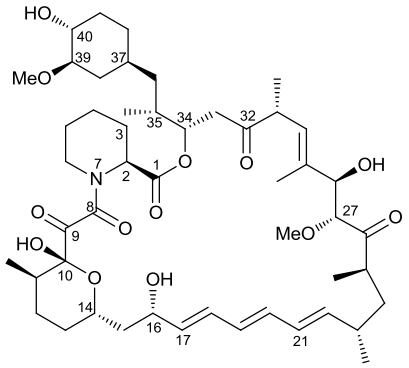
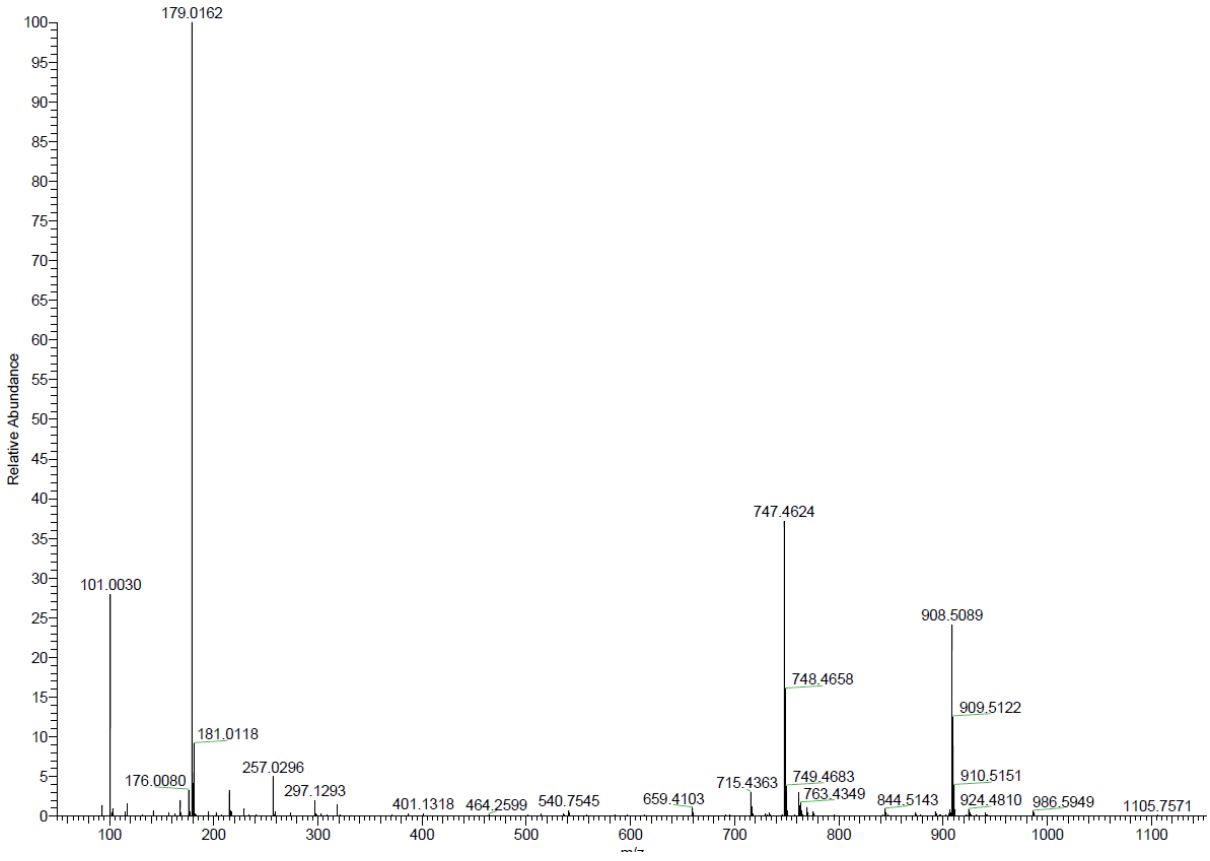
16-*O*-desmethyl-17-desmethylrapamycin (8)



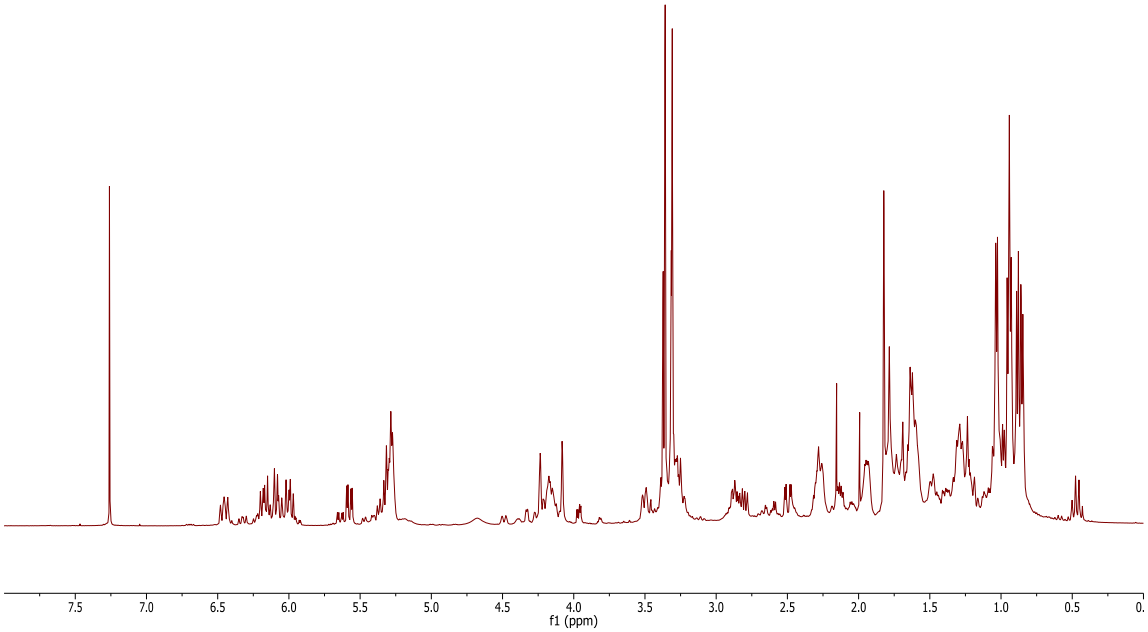
λ_{max} = 270 nm (triplet centred at 270 nm, peaks at 260 nm, 280 nm)

$C_{49}H_{75}NNaO_{13}$, calculated: 908.5131; found: 908.5089; Δ = -4.58 ppm

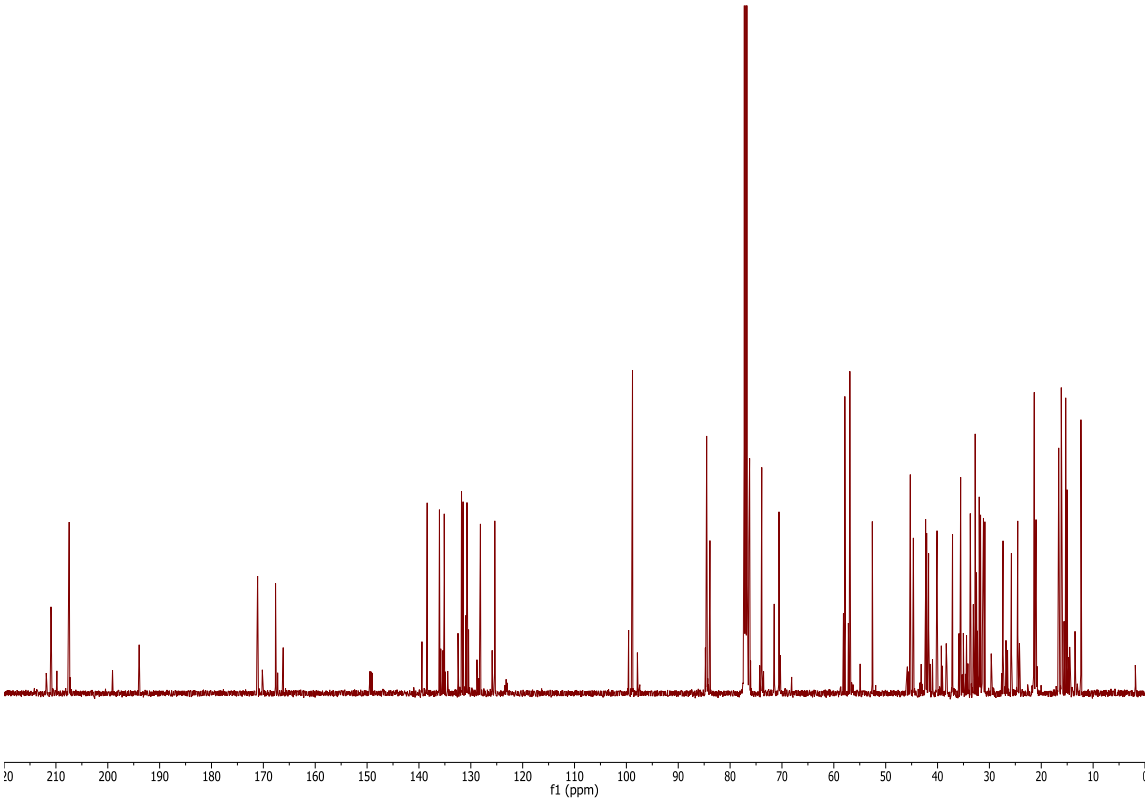


Electronic Supplementary Information

^{1}H-NMR

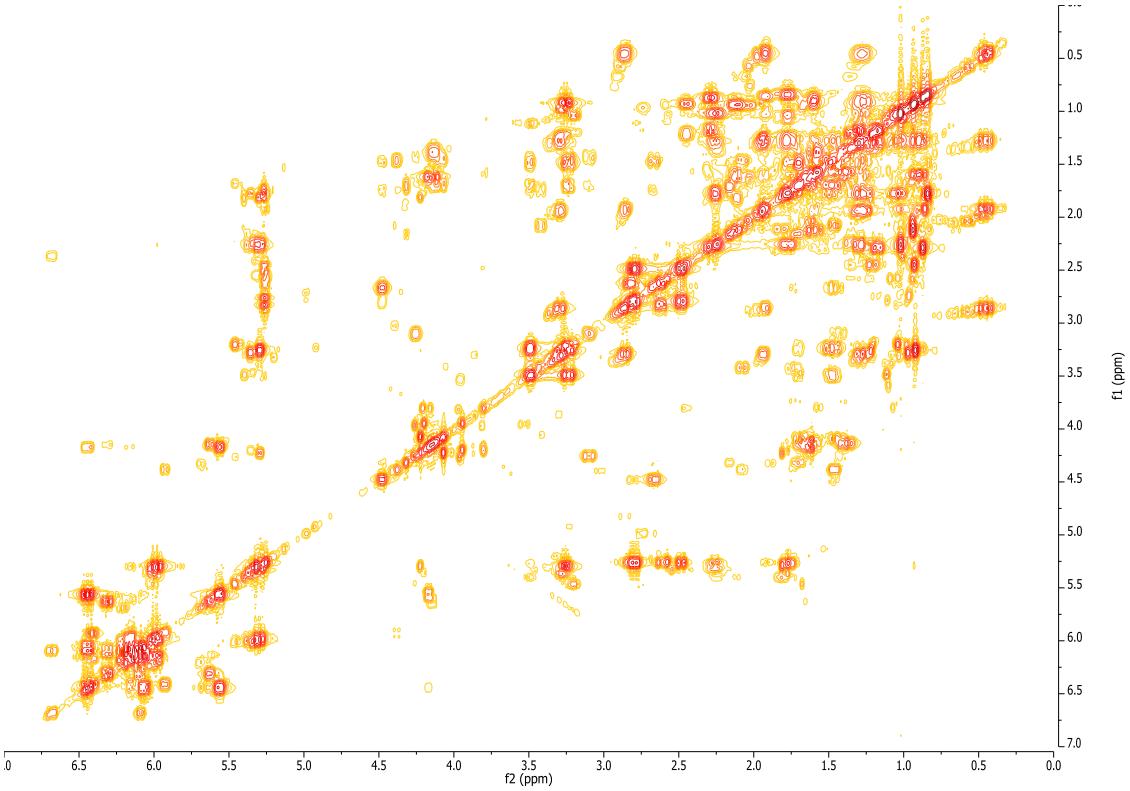


^{13}C-NMR

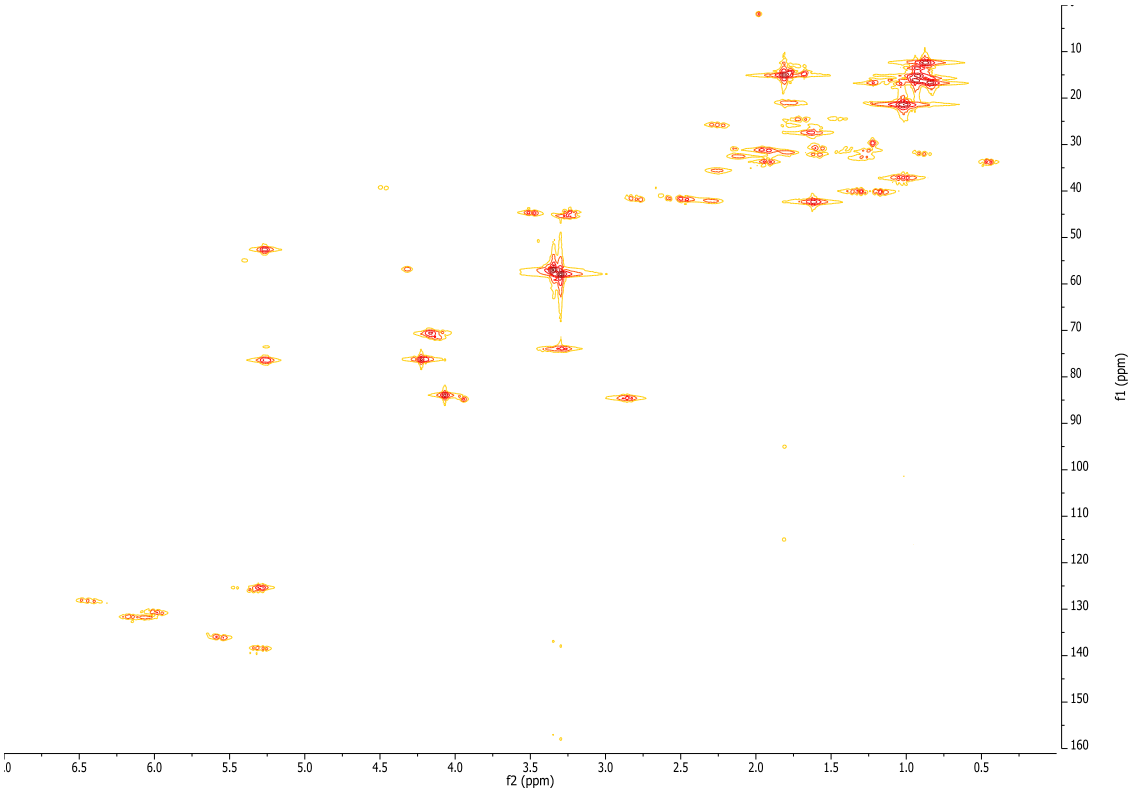


Electronic Supplementary Information

^{1}H-^{1}H-COSY



HMBC



Electronic Supplementary Information

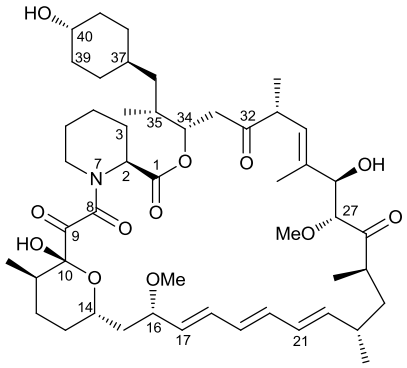
HMQC

Electronic Supplementary Information

Position	δ_H ppm	Multiplicity, Hz	δ_C ppm
1	-	-	171.1
2	5.27	br. d, 5	52.5
3	2.28	m, complex	27.3
4	1.93	m, complex	21.0
	1.48	m, complex	
5	1.75	m, complex	25.2
	1.42	m, complex	
6	2.65	ddd, 16, 10.5, 5	44.6
	2.59	ddd, 16, 9.5, 6	
7	-	-	N
8	-	-	167.6
9	-	-	193.9
10	-	-	98.8
10-OH	4.68	br. s	O
11	2.13	ddq, 11.5, 4, 6.5	32.4
11-CH_3	0.85	d, 6.5	16.6
12	1.69	m, complex	24.5
	1.45		
13	2.14	m, complex	30.8
	1.31	m, complex	
14	4.16	m, complex	70.5
15	2.78	ddd, 16, 10.5, 11	41.7
	2.49	ddd, 16,5.5, 6	
16	4.21	ddd, 8, 5.5, 5.5	76.3
16-OH	3.36	br. s	O
17	5.57	dd, 10.5, 8	136.0
18	6.08	dd, 11, 10.5	131.7
19	6.17	dd, 14.5, 11	131.7
20	5.33	dd, 14.5, 10.5	125.3
21	6.45	dd, 15, 10.5	128.7
22	5.31	dd, 15, 8	138.4
23	2.26	m, complex	35.5
23-CH_3	1.02	d, 6.5	21.3
24	1.18	m, complex	40.0
	1.30	m, complex	
25	1.61	ddq, 10.5, 6.5, 4	42.0
25-CH_3	0.88	d, 6.5	12.3
26	-	-	210.9
27	4.07	d, 4	83.8
27-OCH_3	3.31	s	57.8
28	4.23	d, 4	76.2
28-OH	3.51	br. s	O
29	-	-	135.1
29-CH_3	1.82	s	16.1
30	5.99	d, 11	130.7
31	3.27	m, complex	45.2
31-CH_3	0.94	d, 6.5	15.2
32	-	-	207.4
33	2.86	dd, 17.5, 5.5	42.3
	2.49	dd, 17.5, 4	
34	5.27	ddd, 7, 5.5, 4	73.8
35	1.29	m, complex	32.7
35-CH_3	0.96	d, 6.5	15.0
36	1.03	m, complex	37.1
37	1.04	m, complex	31.7
38	1.93	ddd, 16.5, 5, 4	33.6
	0.46	ddd, 16.5, 10, 13.5	
39	2.84	ddd, 13.5, 12.5, 5	84.5
39-OCH_3	3.36	s	56.9
40	4.15	m, complex	71.4
40-OH	3.49	br. s	O
41	1.62	m, complex	31.1
	1.28		
42	1.78	m, complex	31.9
	1.64	m, complex	

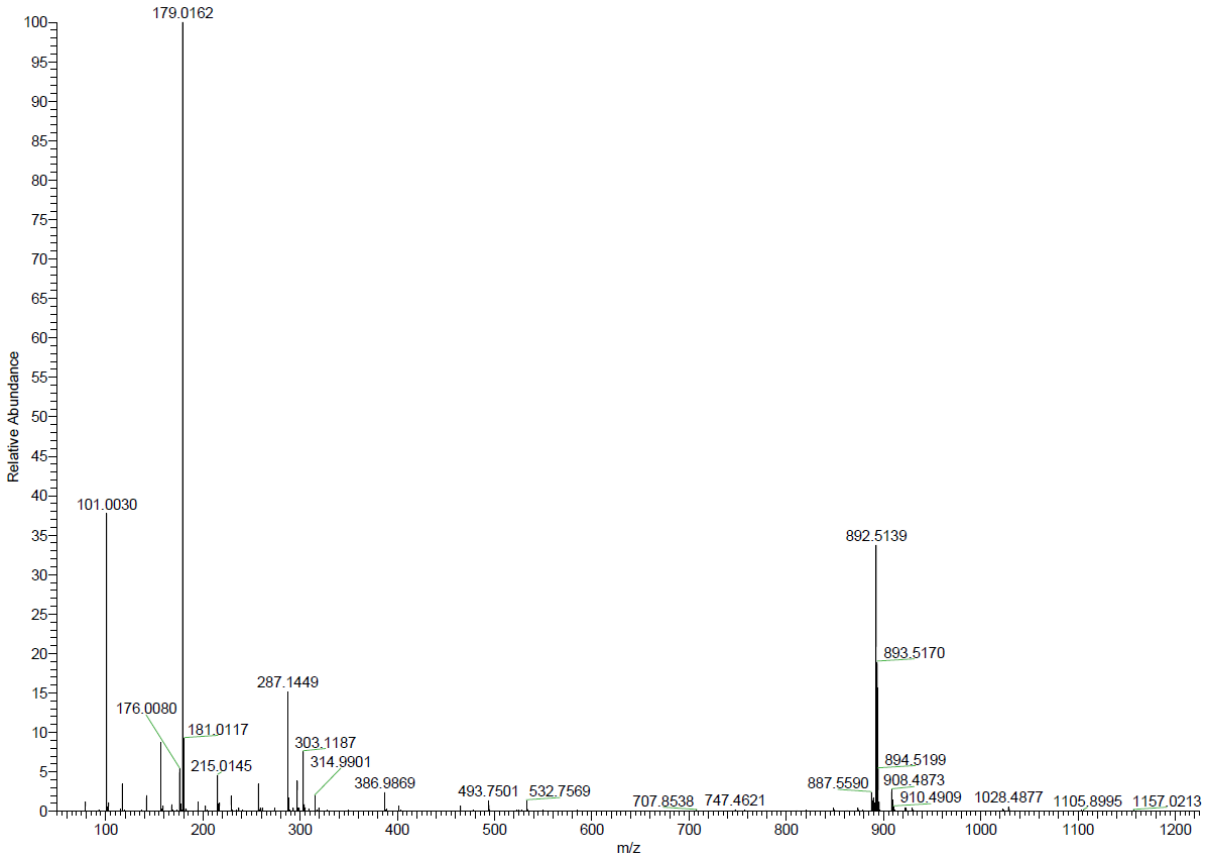
Electronic Supplementary Information

17-desmethyl-39-desmethoxyrapamycin (10)



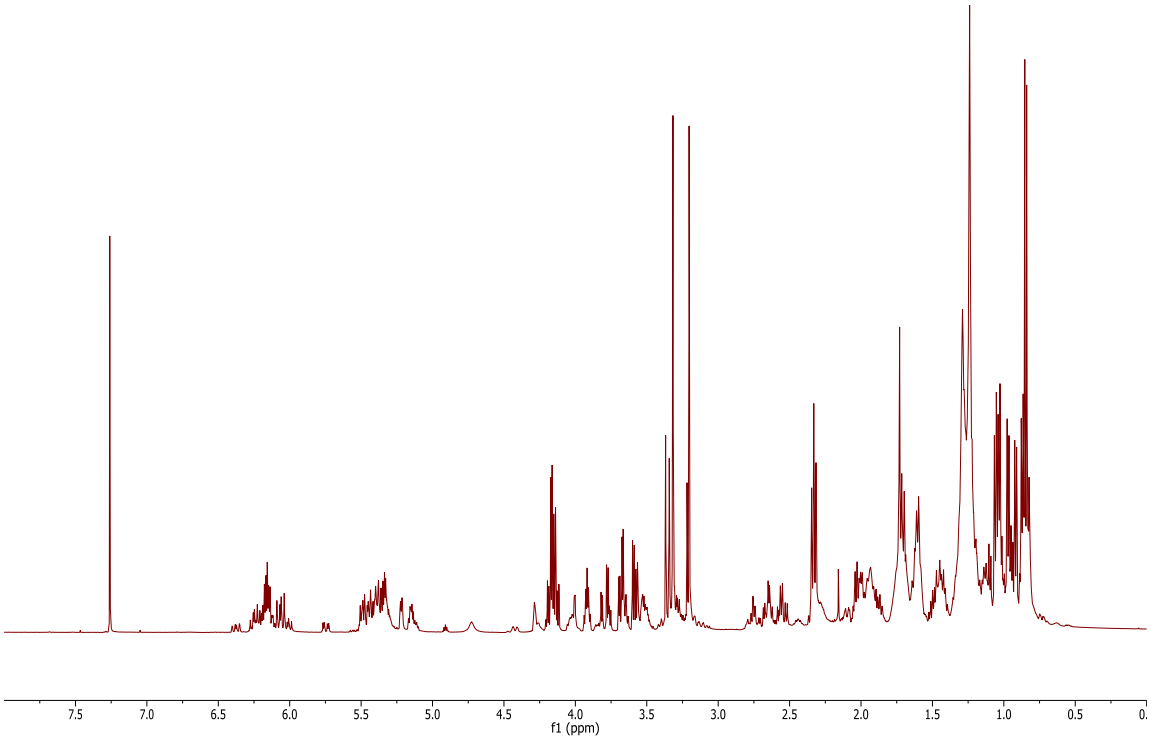
λ_{max} = 270 nm (triplet centred at 270 nm, peaks at 260 nm, 280 nm)

$C_{49}H_{75}NNaO_{12}$ calculated: 892.5181; found: 892.5139; Δ = -4.78 ppm

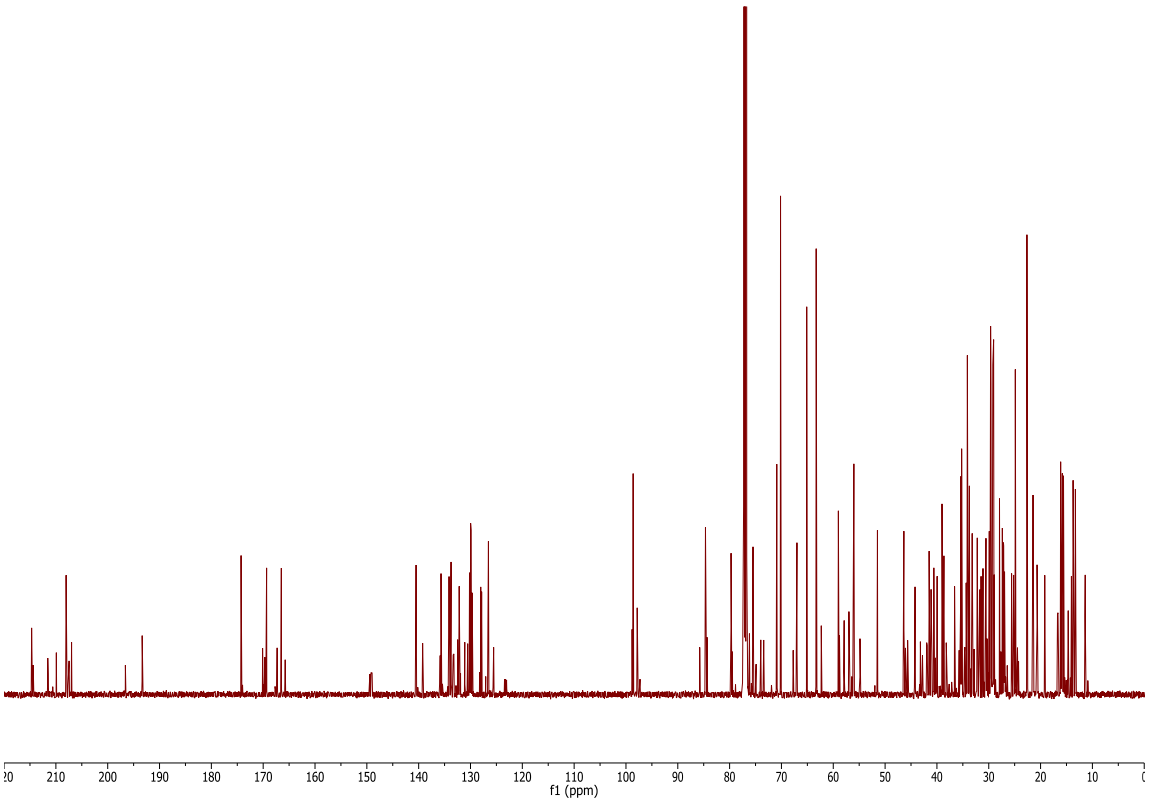


Electronic Supplementary Information

^{1}H-NMR

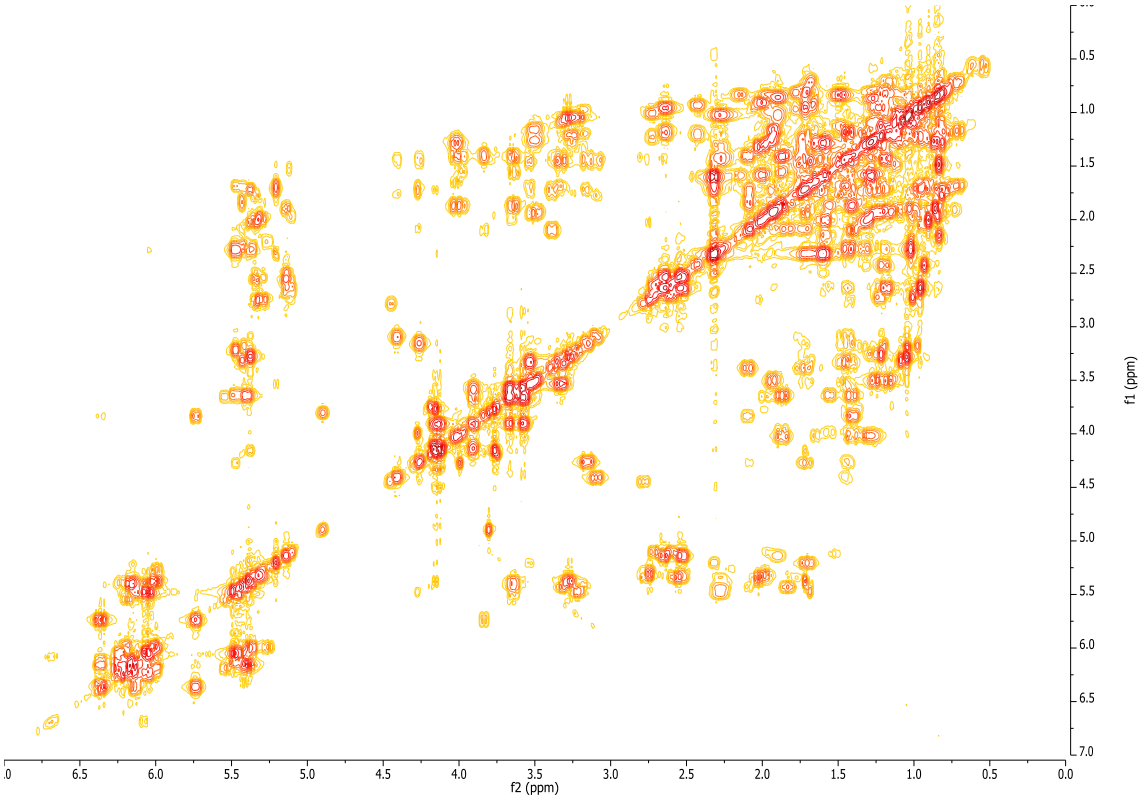


^{13}C-NMR

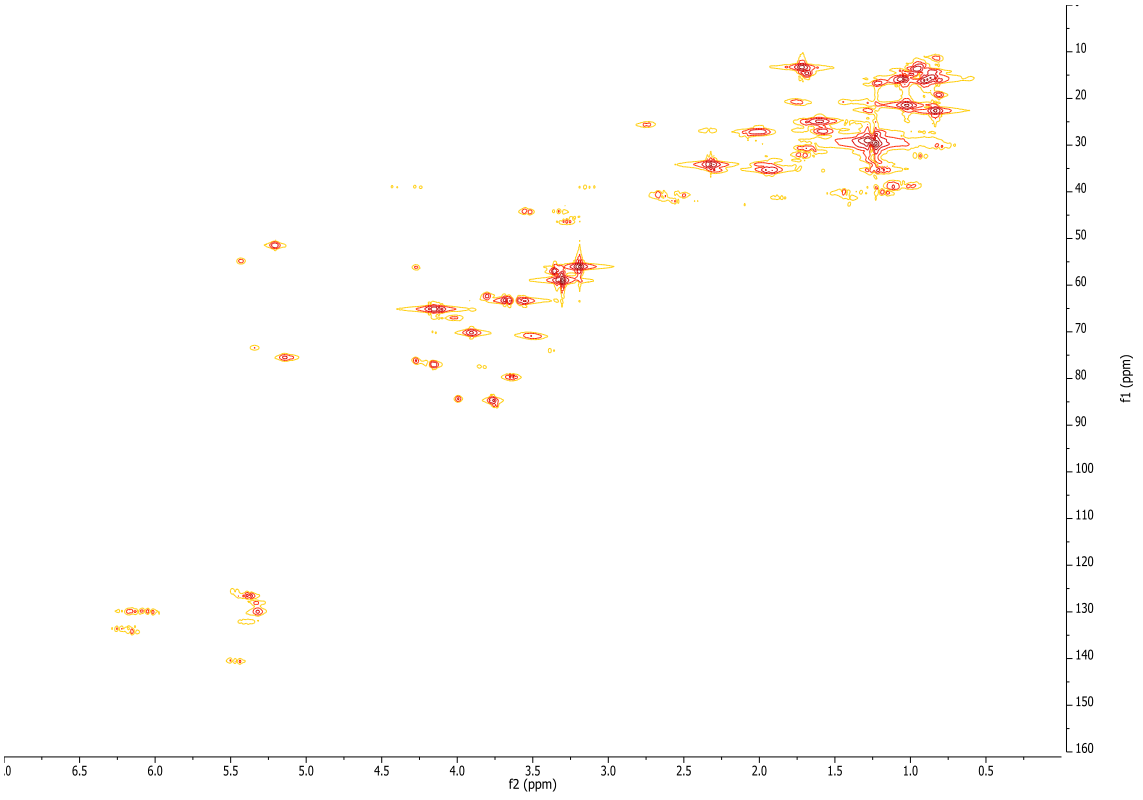


Electronic Supplementary Information

^{1}H-^{1}H-COSY

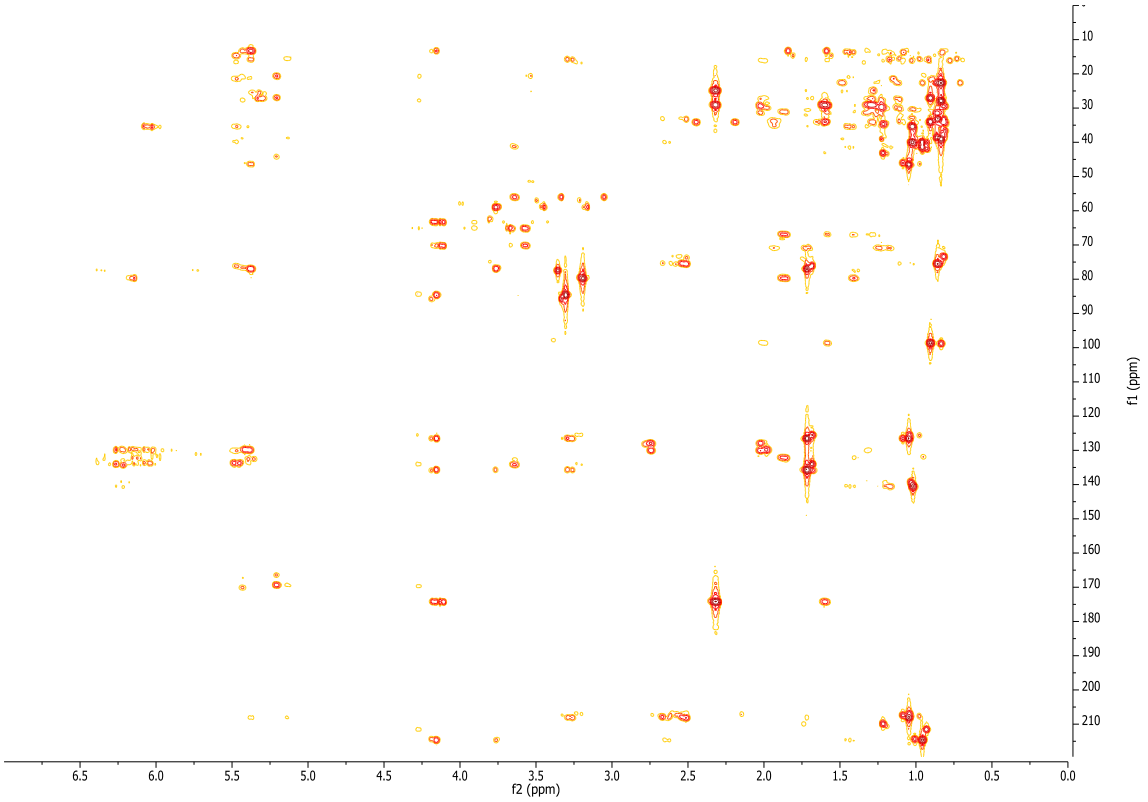


HMBC



Electronic Supplementary Information

HMQC



Electronic Supplementary Information

Position	δ_H ppm	Multiplicity, Hz	δ_C ppm
1	-	-	169.3
2	5.20	br. d, 5	51.4
3	2.35	m, complex	27.3
	1.45		
4	1.88	m, complex	20.6
	1.46	m, complex	
5	1.70	m, complex	25.5
	1.55	m, complex	
6	2.65	ddd, 16, 10.5, 5	44.2
	2.51	ddd, 16, 9.5, 6	
7	-	-	N
8	-	-	166.4
9	-	-	193.3
10	-	-	98.5
10-OH	4.70	br. s	O
11	1.59	ddq, 11.5, 4, 6.5	33.1
11-CH_3	1.02	d, 6.5	16.1
12	1.50	m, complex	27.1
13	1.97	m, complex	31.1
	1.67	m, complex	
14	4.15	m, complex	66.9
15	1.48	ddd, 16, 10.5, 11	39.9
	1.11	ddd, 16,5.5, 6	
16	3.90	ddd, 8, 5.5, 5.5	84.5
16-OCH_3	3.29	s	56.0
17	5.43	dd, 10.5, 8	134.1
18	6.15	dd, 11, 10.5	132.1
19	6.21	dd, 14.5, 11	133.7
20	6.09	dd, 14.5, 10.5	126.5
21	6.06	dd, 15, 10.5	129.9
22	5.33	dd, 15, 8	140.4
23	2.31	m, complex	35.2
23-CH_3	0.94	d, 6.5	21.4
24	1.45	m, complex	40.5
	1.15	m, complex	
25	1.99	ddq, 10.5, 6.5, 4	41.1
25-CH_3	0.90	d, 6.5	13.6
26	-	-	214.7
27	3.65	d, 4	79.7
27-OCH_3	3.19	s	59.0
28	4.13	d, 4	75.4
28-OH	4.52	br. s	O
29	-	-	135.6
29-CH_3	1.73	s	13.2
30	5.40	d, 11	129.8
31	2.74	dq, 11, 6.5	46.3
31-CH_3	1.12	d, 6.5	15.7
32	-	-	208.0
33	2.74	dd, 17.5, 5.5	41.5
	2.52	dd, 17.5, 4	
34	5.13	ddd, 7, 5.5, 4	77.1
35	1.60	m, complex	31.4
35-CH_3	0.84	d, 6.5	15.5
36	1.60	m, complex	38.6
37	1.67	m, complex	34.3
38	2.03-1.11	m, complex	34.1
39	1.93	m, complex	35.4
	1.20		
40	3.90	m, complex	77.8
40-OH	4.28	br. s	O
41	1.93	m, complex	35.4
	1.20		
42	2.03-1.11	m, complex	34.1

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